PEX5, the shuttling import receptor for peroxisomal matrix proteins, is a redox-sensitive protein

Oksana Apanasets[1], Cláudia P. Grou[2], Paul P. Van Veldhoven[1], Chantal Brees[1], Bo Wang[1], Marcus Nordgren[1], Gabriele Dodt[3], Jorge E. Azevedo[2,4], Marc Fransen[1,*]

[1] Laboratory of Lipid Biochemistry and Protein Interactions, Department of Cellular and Molecular Medicine, KU Leuven, Belgium
[2] Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Portugal
[3] Interfakultäres Institut für Biochemie, Universität Tübingen, Germany
[4] Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Portugal

[*] Corresponding author. Tel.: +32 16 330114; Fax: +32 16 330642; E-mail address: marc.fransen@med.kuleuven.be

Running title: PTS1 protein import and oxidative stress

Synopsis: Mounting evidence suggests that oxidative stress impairs peroxisomal matrix protein import. This study provides direct evidence that the import of PTS1 matrix proteins into peroxisomes is indeed a redox-regulated process, and that this redox-sensitivity is mediated by PEX5, the shuttling receptor for PTS1 proteins. In addition, it demonstrates that Cys11 of human PEX5 functions as a redox switch that modulates PEX5 activity in response to intracellular oxidative stress, most likely by modulating its monoubiquitination at the peroxisomal membrane.

Key words: peroxisomes, protein import, PTS1, PEX5, monoubiquitination, redox switch, oxidative stress

Abbreviations: DTM, docking/translocation machinery; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; GST-Ub, GST-tagged ubiquitin; HuFs, human fibroblasts; MEMα, minimum essential medium Eagle alpha; NAC, N-acetyl-cysteine; NEM, N-ethylmaleimide; PNS, postnuclear supernatant; REM, receptor export module; roGFP2, redox-sensitive variant of the enhanced green fluorescent protein; ROS, reactive oxygen species.

Abstract

Peroxisome maintenance depends on the import of nuclear-encoded proteins from the cytosol. The vast majority of these proteins is destined for the peroxisomal lumen and contains a C-terminal peroxisomal targeting signal, called PTS1. This targeting signal is recognized in the cytosol by the receptor PEX5. After docking at the peroxisomal membrane and release of the cargo into the organelle matrix, PEX5 is recycled to the cytosol through a process requiring monoubiquitination of an N-terminal, cytosolically-exposed cysteine residue (Cys11 in the human protein). At present, the reason why a cysteine, and not a lysine residue, is the target of ubiquitination remains unclear. Here, we provide evidence that PTS1 protein import into human fibroblasts is a redox-sensitive process. We also demonstrate that Cys11 in human PEX5 functions as a redox switch that regulates PEX5 activity in response to intracellular oxidative stress. Finally, we show that exposure of human PEX5 to oxidized glutathione results in a ubiquitination-deficient PEX5 molecule, and that substitution of Cys11 by a lysine can counteract this effect. In summary, these findings reveal that the activity of PEX5, and hence PTS1 import, is controlled by the redox state of the cytosol. The potential physiological implications of these findings are discussed.

Introduction

Peroxisomes are multifunctional organelles that, among other functions, participate in cellular lipid and reactive oxygen species (ROS) metabolism (1,2). Defects in peroxisome function are at the core of several fatal genetic disorders (3). In addition, there is growing evidence that peroxisome biology and cellular aging are closely intertwined (4). For example, inhibition of the activity of catalase, the most abundant peroxisomal antioxidant enzyme, causes a cascade of accelerated aging reactions such as increased mitochondrial ROS production, oxidative damage to proteins and DNA, hyper-secretion of matrix metalloproteinases, and impaired cell growth (5,6). On the other hand, increased cellular oxidative stress (e.g. during cellular aging) leads to a reduced import efficiency of peroxisomal matrix proteins containing a C-terminal peroxisomal targeting signal type 1 (PTS1), especially catalase (7). The molecular mechanisms underlying these phenomena are far from being understood. However, in this context, it should be mentioned that (i) under conditions of oxidative stress, PEX5, the shuttling import receptor for PTS1 proteins, accumulates at the peroxisome membrane, and (ii) the cellular senescence phenotypes in old cells can be reversed by overexpression of catalase-SKL, a catalase derivative with enhanced peroxisome targeting efficiency (8).

The assembly of functional peroxisomes requires the import of both membrane and matrix proteins (9). The vast majority of matrix proteins contain a PTS1. This class of proteins is synthesized on free polyribosomes in the cytosol and posttranslationally recognized by the shuttling import receptor PEX5 while still in the cytosol. The PEX5-cargo protein complex then interacts with the peroxisomal docking/translocation machinery (DTM). This interaction ultimately leads to the insertion of PEX5 into the DTM with the concomitant translocation of the cargo protein across the peroxisomal membrane. Interestingly, a 2-kDa N-terminal region

of PEX5 containing a phylogenetically conserved cysteine residue remains exposed to the cytosol during this process. After cargo release, PEX5 is monoubiquitinated at this conserved cysteine residue (Cys11), and extracted from the DTM by the ATP-dependent receptor export module (REM) (10-12). Once back in the cytosol, the ubiquitin moiety attached to PEX5 is removed, probably by a combination of enzymatic and non-enzymatic mechanisms (13,14). This resets PEX5 for the next round of import.

Protein ubiquitination is a posttranslational modification that controls many intracellular processes (15,16). In general, this modification results in the attachment of a ubiquitin molecule to an ε-amino group of a substrate lysine residue (17). However, in a select set of proteins (e.g. PEX5), ubiquitin is transferred onto a cysteine residue yielding a thioester (18). The physiological relevance and role of this unconventional type of ubiquitination is puzzling. On one hand, it is well known that thioesters are easily disrupted by nucleophiles such as GSH (19). On the other hand, it has been reported that substitution of the cysteine at position 11 by lysine in human PEX5 results in a fully functional PTS1 import receptor, at least under standard laboratory conditions (13). Prompted by the combined observations that (i) PEX5 accumulates at the peroxisomal membrane upon oxidative stress (8), (ii) this peroxin needs to be monoubiquitinated at a conserved cysteine residue in order to be exported back to the cytosol (20,21), and (iii) there is an ever increasing number of examples in which the activity of proteins (e.g. transcription factors, kinases, phosphatases, etc.) is controlled by the oxidation states of cysteines (22), we recently hypothesized that Cys11 in human PEX5 may serve as a functional redox switch regulating the peroxisomal/cytosolic localization of peroxisomal proteins such as catalase (2). In this report, we provide experimental evidence in favor of this hypothesis.

Results

Redox regulation of PTS1 protein import into peroxisomes

Previously, we and others have shown that there is a strong correlation between cellular aging, oxidative stress, and PTS1 protein import into peroxisomes (7,8,23). To strengthen the concept that changes in the cellular redox state may have wide-ranging consequences for peroxisomal matrix protein import, we cultivated human fibroblasts (passage 35) under different redox conditions and studied the subcellular localization of roGFP2-PTS1, a peroxisome-targeted variant of roGFP2 (Fig. 1A). RoGFP2 is a redox-sensitive form of the enhanced green fluorescent protein (EGFP) that contains two engineered cysteine residues on adjacent surface-exposed strands close to the chromophore (24). As (i) roGFP2 has two fluorescence excitation maxima at ~400 and ~490 nm, and (ii) disulfide bond formation between the engineered cysteine residues leads to an increase in the excitation peak at ~400 nm at the expense of the ~490 nm peak, the ratio of roGFP2 emissions (at ~510 nm) can provide a non-destructive read-out of the redox environment of the chromophore (24). To determine the effects of different treatments on the redox state of the cytosol, we employed non-targeted, i.e. cytosolic, roGFP2 (23). We found that overexpression of catalase, a peroxisomal H_2O_2-decomposing enzyme whose activity drops during *in vitro* cellular aging (25), significantly lowers the redox state of the cytosol (Fig. 1B) and largely restores PTS1 protein import (Fig. 1C). A similar result was obtained when the cells were cultivated overnight in medium containing 0.5 mM dithiothreitol (DTT), a membrane-permeable reducing agent (Figs. 1B and 1C). For comparison, we also included the values we obtained for these fibroblasts at passage 15 (Figs. 1B and 1C). Taken together, these findings lend support to the idea that the import of PTS1 proteins into peroxisomes is a redox-regulated process.

The redox-dependent changes in PTS1 import efficiency involve the evolutionarily conserved cysteine residue in the N-terminal domain of PEX5

As (i) PTS1 proteins are imported into peroxisomes by the shuttling receptor PEX5 (9), (ii) this receptor accumulates at the peroxisomal membrane in aging cells (7), and (iii) the export of PEX5 from the peroxisomal membrane to the cytosol depends on the evolutionarily conserved cysteine residue in the N-terminal region of the protein (20), we investigated whether or not this cysteine can act as a redox-sensitive regulatory switch for PEX5 activity. Therefore, we complemented late-passage primary human fibroblasts (HuFs) lacking functional PEX5 (hereafter called PEX5 null; for more details, see "Materials and Methods") with constructs encoding human $PEX5_{WT}$ or $PEX5_{C11K}$ and determined how efficiently the expression of both PEX5 variants restored PTS1 protein import. The reasoning behind this experiment was that – in case Cys11 does indeed function as a redox switch – $PEX5_{C11K}$, a peroxisomal import/export-competent protein (13), should restore PTS1 import more efficiently than $PEX5_{WT}$ in cells with an increased cytosolic redox state. To exclude the possibility that any observed differences are the indirect result of non-redox regulated events, we also performed similar experiments in the corresponding transformed HuFs, which display a lower cytosolic redox state (see below). The results of these experiments are presented in Fig. 2A. From these data, it is clear that (i) the import of roGFP2-PTS1 into peroxisomes is PEX5-dependent, (ii) $PEX5_{WT}$ is less functional in late-passage primary cells than in transformed cells, and (iii) the replacement of cysteine at position 11 by a lysine in PEX5 dramatically increases its functionality in late-passage primary cells. To provide unambiguous evidence that the differences observed in primary and transformed cells expressing $PEX5_{WT}$ and $PEX5_{C11K}$ are redox-related and not simply due to differences in expression or stability of these PEX5 proteins, we confirmed that transformation of the

PEX5 null HuFs led to a more reduced redox state of the cytosol (Fig. 2B) and studied the effect of the thiol-reducing agent N-acetyl-cysteine (NAC) on the complementation activity of $PEX5_{WT}$ and $PEX5_{C11K}$ (Fig. 3). Importantly, treatment of the cells with NAC, a compound that shifts the redox state of the cytosol toward lower values (26) (Fig. 3A), resulted in an increased functionality of $PEX5_{WT}$, but not of $PEX5_{C11K}$ (Figs 3B and 3C). Note that the results obtained for $PEX5_{C11K}$ also exclude the possibility that the differences in import kinetics observed for $PEX5_{WT}$ upon NAC treatment are the result of redox-related structural changes of the roGFP2-PTS1 reporter protein. All together, these experiments suggest that Cys11 of human PEX5 functions as a redox switch that modulates PEX5 activity in response to intracellular oxidative stress.

Exposure of human PEX5 to oxidized glutathione impairs its monoubiquitination in a Cys11-dependent manner

To determine whether or not oxidation of human PEX5 affects its monoubiquitination at Cys11, we incubated ^{35}S-labeled $PEX5_{WT}$ or $PEX5_{C11K}$ with a (peroxisome-containing) rat liver postnuclear supernatant supplemented with ATP and GST-tagged ubiquitin (GST-Ub) in the absence or presence of increasing concentrations of oxidized glutathione (GSSG) (for detailed procedures, see "Materials and Methods"). Organelles were then processed for SDS-PAGE under non-reducing and reducing conditions, and the amounts of ^{35}S-labeled GST-Ub-PEX5 species that were produced in these in vitro assays were assessed by autoradiography (Fig. 4). As shown in Fig. 4 (upper panel), monoubiquitination of $PEX5_{WT}$ is much more sensitive to GSSG than monoubiquitination of $PEX5_{C11K}$. Note that PEX5 monoubiquitination detected in these assays occurs at its residue 11, because treatment of protein samples with DTT destroys the thioester bond between GST-Ub and $PEX5_{WT}$ but not the amide bond that links GST-Ub to $PEX5_{C11K}$ (Fig. 4, lower panel; see also (13)). The reduction in $PEX5_{C11K}$ monoubiquitination observed with the highest concentration of GSSG (Fig. 4, upper panel) is probably due to the fact that the ubiquitination cascade comprises cysteine-containing enzymes (the ubiquitin-activating enzyme and ubiquitin-conjugating enzymes) that are also potential targets of GSSG (27). In summary, these data strongly indicate that Cys11 in human PEX5 is the most oxidative stress-sensitive cysteine of the complete PEX5 monoubiquitination process.

Discussion

In this study, we have provided experimental evidence that (i) PTS1 protein import is a redox-sensitive process, (ii) this redox sensitivity is mediated by PEX5, the shuttling receptor for peroxisomal matrix proteins, and (iii) the cysteine at position 11 in human PEX5 functions as a redox switch that regulates PEX5 activity in response to oxidative stress, most likely by modulating its monoubiquitination at the DTM. In summary, these findings provide a molecular explanation for the long-standing observation that peroxisomal PTS1 protein import is reduced in cells experiencing oxidative stress (7,28). In addition, as PEX5 monoubiquitination at Cys11 is necessary for the export of the receptor back into the cytosol (20,21), these data also explain why PEX5 accumulates on peroxisomal membranes during cellular aging (7), a condition associated with a sharp increase in the cytosolic redox state (23,29). In the context of these results, it is important to recognize that also additional mechanisms may be at play in relation to the inhibitory effects of oxidative stress on peroxisomal protein import. For example, as oxidative stress can decrease both glycolytic and mitochondrial ATP production (30, 31), it is very likely that this type of stress can also impede the ATP-dependent export step of PEX5 from the peroxisomal membrane to the cytosol. In addition, one can easily envisage a scenario in which oxidative stress leads to the modification and partial inactivation of non-PEX5-related components of the peroxisomal protein import and protein quality control machineries (32,33). However, as (i) such alternative mechanisms can be expected to equally reduce the functionality of $PEX5_{WT}$ and $PEX5_{C11K}$, and (ii) this is not the case in the experiments discussed here, they seem to be of no importance under the conditions studied.

Over the years, accumulating evidence has strengthened the view that peroxisomal metabolism, cellular oxidative balance, and cellular aging are intimately interconnected (2,4,34). In the context of this work, it is important to remember that Terlecky and colleagues in 2006 postulated the existence of a self-sustaining peroxisome deterioration spiral (35). According to this model, peroxisomes are predisposed to oxidative stress due to a dysregulated import of peroxisomal H_2O_2-producing oxidases, which in general bear a strong PTS1 (36), and catalase, which contains a weak PTS1 (37). This causes the organelle to slowly lose its ability to clear generated ROS, and – as such – its import machinery may become gradually compromised. This, in turn, will initially mainly affect the import of weak substrates, such as catalase, but later on result in a self-perpetuating negative protein import spiral causing oxidative stress and peroxisome dysfunction (6,35). The data presented in this manuscript extend this theory by providing specific details regarding the potential mechanisms by which cellular oxidative stress causes peroxisomal protein import defects. Note that, as the monoubiquitinable cysteine residue in the N-terminus of PEX5 and the weak PTS1 of catalase are highly conserved in virtually all mammalian species (data not shown), it is tempting to speculate that these features have evolved because of a possible advantage. For example, a decrease in the peroxisomal import efficiency of (newly-synthesized) catalase may allow cells to rapidly respond to oxidative stress in the cytosol. This postulate is in line with a recent study showing that p97, an ATPase of the AAA family (ATPases with diverse cellular activities), can regulate H_2O_2 levels in the cytosol by affecting the

retention time of (newly-synthesized) catalase within this subcellular compartment (38).

Importantly, when the work presented in this manuscript was in a final stage, Subramani and coworkers reported that also *Pichia pastoris* Pex5 (PpPex5) functions as a redox-regulated PTS1 protein import receptor (39). Specifically, these authors reported that Cys10, the evolutionarily conserved redox-sensitive cysteine in PpPex5, acts as a redox switch to control the binding and release of PTS1 proteins to and from PpPex5 by modulating its oligomeric state. Based on these observations and a report stating that the intraperoxisomal redox state is more reductive than that of the cytosol (40), the authors postulated a working model in which (oxidized) disulfide bond-linked PpPex5 binds newly-synthesized PTS1 proteins in the cytosol and releases these proteins upon reduction in the peroxisomal matrix. However, here it is important to note that this model is not compatible with our data. Indeed, we clearly show that the reduced functionality of PEX5 in an oxidizing environment can be counteracted by substituting Cys11 with lysine, and this finding in turn strongly indicates that – at least for human PEX5 – oxidation-mediated disulfide bond formation through this conserved cysteine can be functionally uncoupled from PTS1 cargo binding and release. In this context, it is also important to point out that some of us have previously shown that, in a mammalian system, (i) PTS1 proteins can be efficiently imported into peroxisomes under conditions in which the redox state of peroxisomes is more oxidizing than that of the cytosol (23), (ii) the release of PTS1 cargo proteins into the peroxisomal matrix is most likely triggered by binding of PEX14 to the 6th (and probably 7th) diaromatic motif(s) of PEX5 (41), and (iii) PEX5$_{C11A}$, a mutant of PEX5 that is not capable of forming a disulfide bond through the conserved cysteine, can translocate PTS1 proteins across the peroxisomal membrane and release these proteins into the organellar matrix as efficient as PEX5$_{WT}$, at least in a cell-free *in vitro* import system (as PEX5$_{C11A}$ cannot be monoubiquitinated at the peroxisomal membrane and hence not be exported back to the cytosol, this protein abrogates PTS1 protein import upon expression in wild-type cells) (21,42). Nevertheless, as yeast and mammalian systems are not always directly comparable because of differences in PEX proteins and enzyme content (9), it remains to be established whether or not the apparently conflicting data obtained for PpPex5p and human PEX5 represent species- or condition-specific characteristics.

In conclusion, our data provide clear evidence that (i) the activity of PEX5 depends on the redox state of the cytosol, and (ii) this redox sensitivity is mediated by Cys11, an evolutionarily conserved amino acid involved in receptor monoubiquitination and recycling. These findings are relevant for a better understanding of how cellular oxidative stress may contribute to a demise of peroxisome function, a condition associated with cellular senescence, organismal aging, and age-related diseases (4).

Materials and Methods

DNA manipulations and plasmids – The mammalian expression vector pIRES2-EGFP and the restriction enzymes were commercially obtained from Westburg (Leusden, The Netherlands). Oligonucleotides (Supplementary Table 1) were synthesized by Integrated DNA Technologies (IDT, Leuven, Belgium). Polymerase chain reactions were routinely carried out by using *Pfx* DNA Polymerase (Invitrogen, Merelbeke, Belgium). The *E. coli* strain *Top10F'* (Invitrogen) was used for all DNA manipulations. The plasmids encoding a cytosolic roGFP2 (roGFP2; pMF1707), peroxisomal roGFP2 (roGFP2-PTS1; pMF1706), or human catalase (pMF1525) have been described elsewhere (23). The pIRES2-roGFP2-PTS1 (pMN8) plasmid was constructed via a three-point ligation of the following fragments: (i) the *Xma*I/*Not*I-restricted backbone fragment of pIRES2-GFP, (ii) a *Xma*I/*Bam*HI-restricted PCR fragment comprising the IRES2 sequence (template: pIRES2-EGFP2; primers: pIRES2fwXmaI and pIRES2rvBamHI), and (iii) the *Bam*HI/*Not*I restricted fragment of pMF1706, which encodes roGFP2-PTS1. The bicistronic constructs encoding roGFP2-PTS1 in combination with the large isoform of HsPEX5$_{WT}$ (pDC2) or HsPEX5$_{C11K}$ (pDC1) were generated by amplifying the corresponding HsPEX5-encoding cDNA fragments by PCR (templates: pMF1678 and pMF1578, respectively (13); primers: HsPex5.1fw and HsPex5.2rv) and cloning the *Bgl*II/*Sal*I-digested PCR products into the *Bgl*II/*Sal*I-restricted pIRES2-roGFP2-PTS1 vector. All plasmids were verified by DNA sequencing (LGC Genomics, Berlin, Germany). The pGEM4® (Promega)-based plasmids encoding the large isoform of human PEX5 and the mutated version of it possessing a lysine at position 11 (PEX5$_{C11K}$) have been previously described (13,43).

Cell culture, transfections and fluorescence microscopy – The primary PEX5 null human fibroblasts (HuFs; cell line PBD005) have been described elsewhere (44,45). Briefly, these cells are homozygous for a mutation (Arg390Ter) in the *PEX5* gene that results in a complete inactivation of PEX5's normal function (44,45). Control HuFs were kindly provided by Dr. D. Cassiman (KU Leuven, Belgium). Where indicated, the cell lines were transformed by introduction of SV40 large T-antigen. Unless specified otherwise, all cell lines were cultivated at 37°C in a humidified 5% CO_2 incubator in minimum essential medium Eagle alpha (MEMα; Lonza, Verviers, Belgium) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 2 mM Glutamax (Invitrogen), and MycoZap (Lonza). The cells were transfected by using the Neon Transfection System (Invitrogen; primary HuFs: 1200 V, 20-ms pulse width, 1 pulse; transformed HuFs: 1300 V, 30-ms pulse width, 1 pulse). Cells for live-cell imaging were seeded and imaged in FD-35 Fluorodish cell culture dishes (World Precision Instruments, Hertfordshire, England). The technical specifications of the objectives, excitation and emission filters, and digital camera have been described elsewhere (46).

Ratiometric redox measurements were performed as described (23). Note that, as the spectral output of the replacement lamps was not always identical, the emission ratios of roGFP2 at the 400- and 480-nm excitation wavelengths were expressed in arbitrary units (a.u.). The Olympus image analysis and particle detection software were used for quantitative image analysis.

Peroxisome-dependent PEX5-ubiquitination assay – ^{35}S-labeled PEX5 and PEX5$_{C11K}$ were synthesized in rabbit reticulocyte lysates using the TNT® quick coupled transcription/translation system (Promega) in the presence of EasyTag™ L-(^{35}S)methionine (specific activity, >1000 Ci/mmol; PerkinElmer Life Sciences) and the pGEM4®-based plasmids referred to above. Rat liver postnuclear supernatant (PNS; 600 µg of protein) was diluted in import buffer (0.25 M sucrose, 50 mM KCl, 20 mM MOPS-KOH pH 7.2, 3 mM $MgCl_2$, 20 µM methionine, and 2 µg/ml N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide) containing 0.3 mM ATP (final volume 80 µl), and primed for import by incubating the samples for 5 min at 37 °C (47). The samples were placed on ice, and incubated for 10 min in the absence or presence of increasing concentrations oxidized glutathione (GSSG; added from a 0.1 M stock solution in 20 mM MOPS-KOH, pH 7.4). In parallel, 1 µl of ^{35}S-labeled PEX5$_{WT}$ or PEX5$_{C11K}$ (diluted 1:20 (v/v) in import buffer) were also treated with the matching GSSG concentration. Next, the diluted lysates were added to the corresponding PNS mixtures, and the samples were incubated for 20 min at 37 °C in the presence of 5 mM ATP and 7 µM glutathione S-transferase (GST)-ubiquitin (20). The reactions were stopped with 20 mM N-ethylmaleimide (NEM; 5 min on ice), diluted with 900 µl of ice-cold SEMK buffer (0.25 M sucrose, 80 mM KCl, 20 mM MOPS-KOH, pH 7.2, 1 mM EDTA-NaOH, pH 8.0), and centrifuged at 16,000 x g for 20 min at 4°C to separate the organelles from soluble proteins. The organelle pellets were then subjected to SDS-PAGE under non-reducing (no DTT) and reducing (0.1 M DTT) conditions, and analyzed by Western blot and autoradiography, as described before (13,20).

Statistical analysis – Statistics were performed on the VassarStats statistical computation website (http://vassarStats.net/). One-way analysis of variance was used to determine the differences among independent groups of numerical values, and individual differences were further explored with a Student's t-test. The significance level was chosen to be $p<0.01$.

Acknowledgments

The authors are grateful to Dr. Ann Moser (Baltimore, USA) for the primary PEX5 null human fibroblasts. This work was supported by grants from the 'Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (Onderzoeksproject G.0754.09)' (to MF and PPVV), by the KU Leuven grants OT/09/045 (to MF and PPVV) and DBOF/10/059 (to PPVV and MF), and by FEDER funds through the Operational Competitiveness Programme – COMPETE and by National Funds through FCT – Fundação para a Ciência e a Tecnologia under the project FCOMP-01-0124-FEDER-019731 (PTDC/BIA-BCM/118577/2010) (to JEA). Marcus Nordgren is supported by a FLOF fellowship from the Department of Cellular and Molecular Medicine, KU Leuven. Bo Wang is a recipient of a DBOF fellowship (DBOF/10/059) from the KU Leuven. Cláudia P. Grou is supported by Fundação para a Ciência e a Tecnologia, Programa Operacional Potencial Humano do QREN, and Fundo Social Europeu.

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Figure legends

Figure 1. The efficiency of PTS1 protein import into peroxisomes is influenced by the cytosolic redox state. Primary human fibroblasts (HuFs) (passages 15 or 35) were transiently transfected with a plasmid encoding peroxisomal (roGFP2-PTS1) or cytosolic (roGFP2) roGFP2, in combination or not with a plasmid encoding wild-type catalase (cat). The cells were cultivated in standard growth medium and, where indicated, supplemented with 0.5 mM DTT (overnight before analysis). (**A**) Subcellular localizations of roGFP2-PTS1 in late passage cells. Note that, as (i) the cytosolic redox state of these cells displays a considerable intercellular variability (23), and (ii) this variability substantially influences the import efficiency of PTS1 proteins (this study), different cells can display a different distribution pattern for roGFP2-PTS1. PO, peroxisomes; C, cytosol; PO/C, peroxisomes and cytosol. Scale bar: 10 µm. (**B**) Box plot representations of the 400/480 nm fluorescence response ratios of roGFP2 at 6 days post-transfection. The results are normalized to the average value of the 'p15' condition, which is set to one arbitrary unit (a.u.). The bottom and top of each box represent the 25^{th} and 75^{th} percentile values, respectively; the horizontal line inside each box represents the median; and the horizontal lines below and above each box denote the mean minus and plus one standard deviation, respectively. The number of randomly chosen measurements (from at least 6 different cells) is indicated below each box plot. The data were statistically compared. (**C**) Distribution pattern of roGFP2-PTS1 at 3 days post-transfection: PO (black boxes), C (white boxes), or PO/C (grey boxes). The number of cells analyzed is indicated above each condition.

Figure 2. The cysteine at position 11 of human PEX5 mediates the oxidative stress-related decline in PTS1 import efficiency. Primary (P; passage number: 24) and SV40 T-antigen-transformed (T) PEX5 null human fibroblasts (HuFs) were transiently transfected with a plasmid encoding peroxisomal (roGFP2-PTS1) or cytosolic (roGFP2) roGFP2, in combination or not with a plasmid encoding $HsPEX5_{WT}$ (5_{WT}) or $HsPEX5_{C11K}$ (5_{C11K}). The cells were cultivated in the standard growth medium and analyzed 3 days post-transfection. (**A**) Subcellular localizations of roGFP2-PTS1: peroxisomes (PO, black boxes), cytosol (C, white boxes), or peroxisomes/cytosol (PO/C, grey boxes); 100 randomly selected cells were analyzed per condition. (**B**) Box plot representations of the 400/480 nm fluorescence response ratios of roGFP2. The results are normalized to the average value of the corresponding late-passage primary cells, which is set to one arbitrary unit (a.u.). The number of randomly chosen measurements (from at least 10 different cells) is indicated below each box plot. For a description of these plots, see legend of Figure 1. The data were statistically compared.

Figure 3. The efficiency of PTS1 protein import complementation in aged primary PEX5 null human fibroblasts is affected by the redox state of the cytosol and the identity of the ubiquitinatable residue near the N-terminus of human PEX5. Primary PEX5 null human fibroblasts (HuFs; passage number: 25) were transiently transfected with a monocistronic plasmid encoding cytosolic roGFP2 (roGFP2) or a bicistronic plasmid encoding peroxisomal roGFP2 (roGFP2-PTS1) and wild-type HsPEX5 (5_{wt}) or $HsPEX5_{C11K}$ (5_{C11K}). After three days, the standard growth medium was replaced by fresh medium supplemented (+NAC) or not (-NAC) with 1 mM N-acetyl-cysteine. The cells were analyzed at the indicated time points. (**A**) Box plot representations of the 400/480 nm fluorescence response ratios of roGFP2. The results are normalized to the average value of the '0 h' condition, which is set to one arbitrary unit (a.u.). The number of randomly chosen measurements (from at least 15 individual cells) is indicated below

each box plot. For a description of these plots, see legend of Figure 1B. (**B**, **C**) Subcellular localization of roGFP2-PTS1 under different treatment conditions (plotted as a function of time). PO, peroxisomal localization; PO/C, mixed peroxisomal and cytosolic localization.

Figure 4. Oxidized glutathione interferes with monoubiquitination of human PEX5 at Cys11. ^{35}S-labeled $PEX5_{WT}$ and $PEX5_{C11K}$ were subjected to *in vitro* import reactions (supplemented with GST-Ub) in the absence or presence of increasing concentrations of oxidized glutathione (GSSG) (for more details, see "Materials and Methods"). After 20 minutes, the samples were treated with NEM, and the organelles were isolated by centrifugation. The organelle fractions were subjected to SDS-PAGE under non-reducing (-DTT) and reducing (+DTT) conditions, and blotted onto a nitrocellulose membrane. The membrane was exposed to an X-ray film. Lane I, 10% of the ^{35}S-labeled PEX5 reticulocyte lysate used in each lane. The numbers to the left indicate the migration of the molecular mass markers (in kDa).

Figure 1

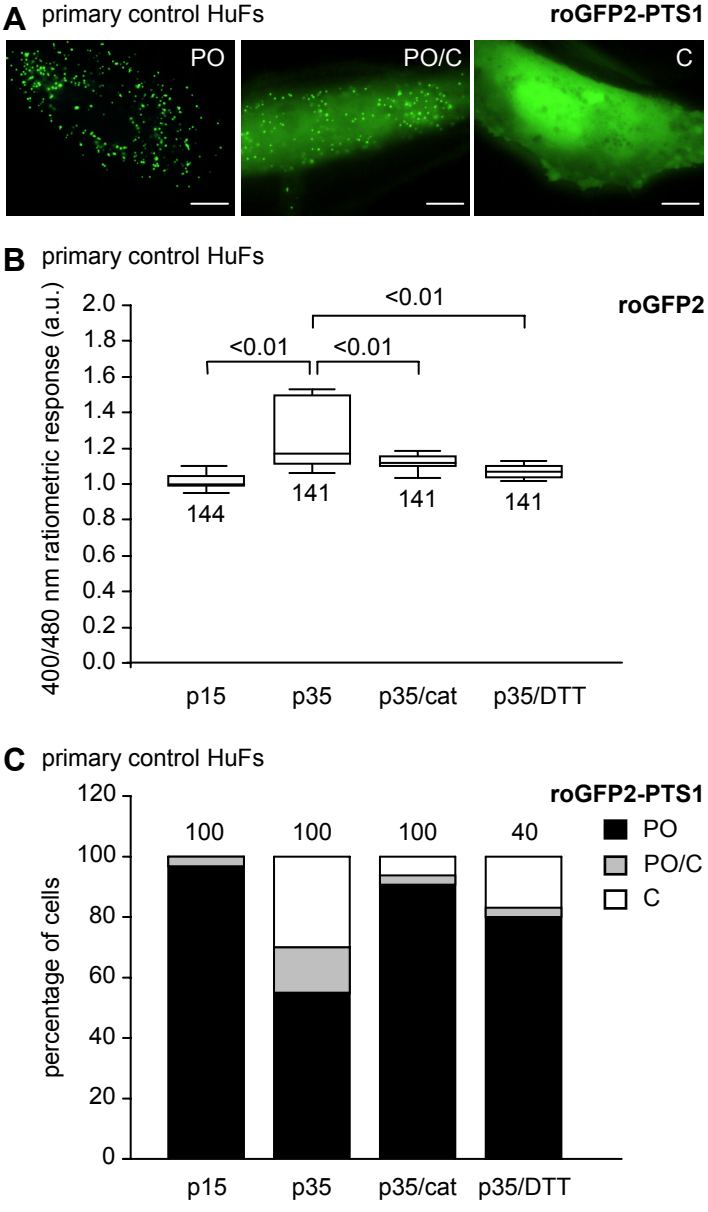

A
primary control HuFs
roGFP2-PTS1
PO
PO/C
C
B
primary control HuFs
roGFP2
400/480 nm ratiometric response (a.u.)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
<0.01
<0.01
<0.01
144
141
141
141
p15
p35
p35/cat
p35/DTT
C
primary control HuFs
roGFP2-PTS1
percentage of cells
120
100
80
60
40
20
0
100
100
100
40
PO
PO/C
C
p15
p35
p35/cat
p35/DTT

Figure 2

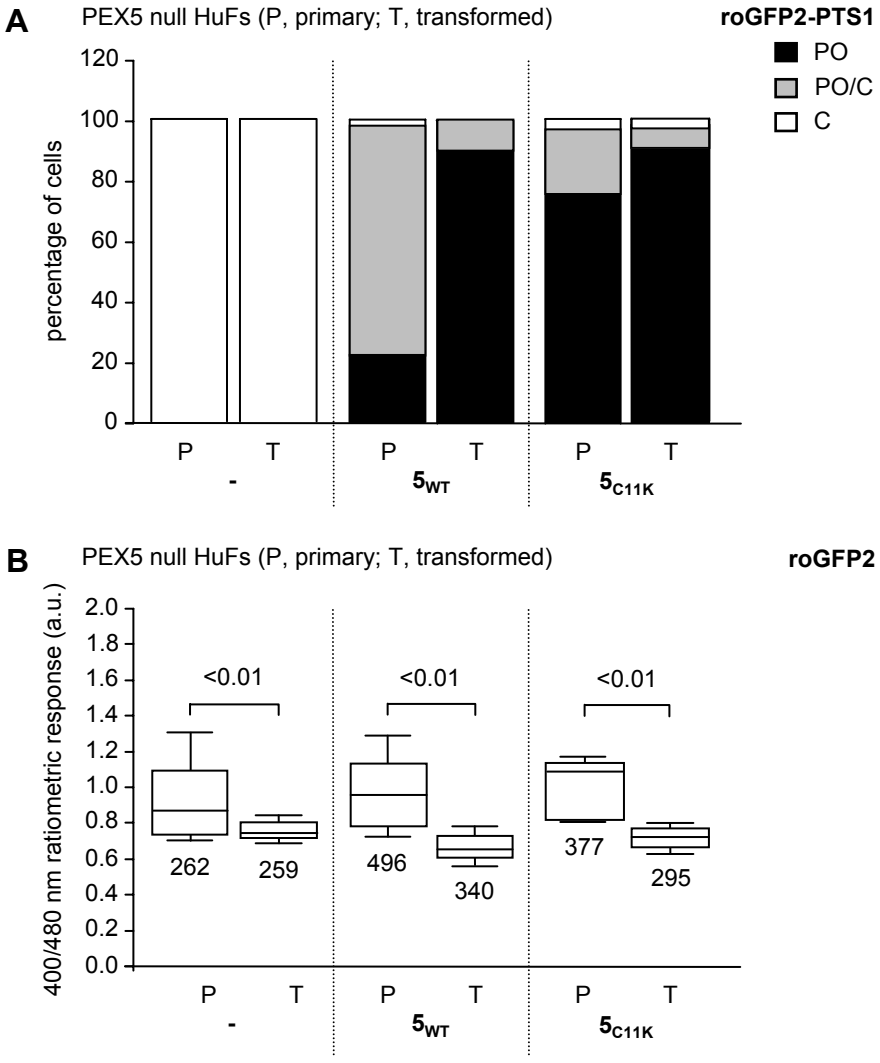

A
PEX5 null HuFs (P, primary; T, transformed)
roGFP2-PTS1
PO
PO/C
C
percentage of cells
120
100
80
60
40
20
0
P
T
-
5WT
5C11K
B
PEX5 null HuFs (P, primary; T, transformed)
roGFP2
400/480 nm ratiometric response (a.u.)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
<0.01
262
259
496
340
377
295

Figure 3

primary PEX5 null HuFs (passage 25)

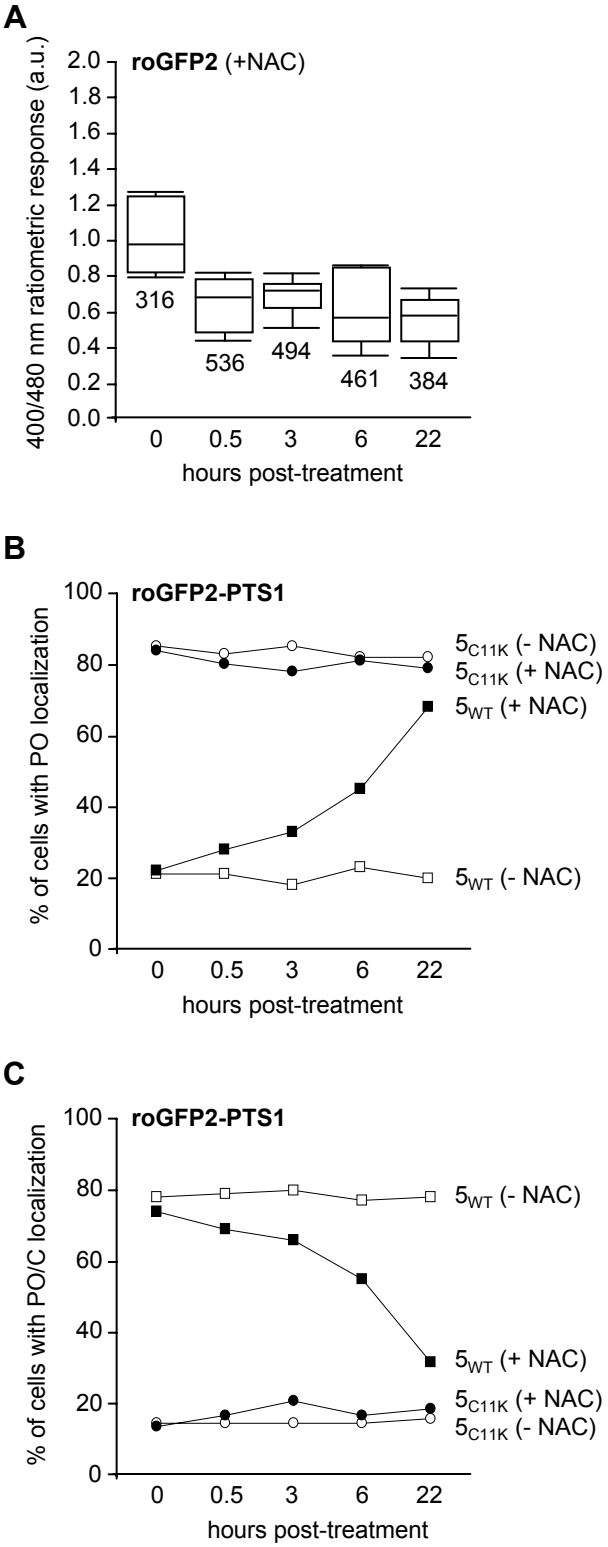
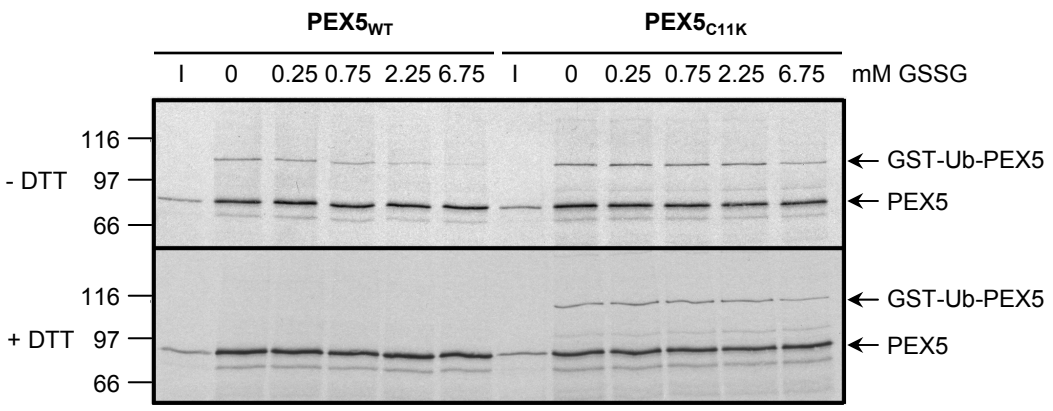


Figure 4


PEX5WT
PEX5C11K
I 0 0.25 0.75 2.25 6.75 I 0 0.25 0.75 2.25 6.75 mM GSSG
- DTT
+ DTT
116
97
66
GST-Ub-PEX5
PEX5

Supplementary Table 1. List of oligonucleotides used in this study*

Name	Nucleotide sequence
HsPex5.1fw	5'- cgagatctgtatggcaatgcgggagctgg -3'
HsPex5.2rv	5'- gcccgtcgacctgtcactggggcaggccaaac -3'
pIRES2fwXmaI	5'- tgaacccgggttccgcccctctccctc -3'
pIRES2rvBamHI	5'- gggggatccttgtggccatattatcatcg -3'

* Restriction sites are underlined

Graphical abstract